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TITLE: Fatty Acid Synthase Activity as a Target for c-Met Driven Prostate Cancer

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14. ABSTRACT - Prostate cancer (PCa) is the most common noncutaneous neoplasia diagnosed in men and represents roughly 10% of cancer-associated mortalities. The lethal phenotype of PCa is primarily characterized by progression of tumor cells to castration-resistance and metastasis. Organ-confined PCa is curable with surgery and/or radiation therapy, however, as the disease becomes metastatic, the likelihood of survival becomes minimal. Key influences in the progression to metastasis are growth factor receptors including the receptor tyrosine kinase c-Met. 14. ABSTRACT During this research period, I have acquired convincing data that the c-Met receptor tyrosine kinase is palmitoylated and that this palmitoylation regulates its stability. Inhibition of palmitoylation reduces the expression of c-Met in prostate cancer cell lines. This protein loss occurs post-transcriptionally and is associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization pathways, as well as surface biotinylation studies and confocal microscopy, we determined that inhibition of palmitoylation reduces the stability of newly synthesized c-Met as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a click-chemistry based palmitate-labeling protocol suggest c-Met itself is palmitoylated. Observing palmitoylation kinetics has provided evidence that c-Met is palmitoylated in the Golgi prior to cleavage of the c-Met precursor. Currently, mass spectrometry analysis is being performed to identify the specific cysteine residue/s that are palmitoylated to aid future studies. Taken together, these findings suggest inhibition of palmitoylation could be a novel target for treating prostate cancer driven by oncogenic c-Met.					
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Fatty Acid Synthase Activity as a Target for c-Met Driven Prostate Cancer

Grant Number: PC102179

Annual Report: 01 July 2011 - 30 June 2012

Introduction

The overarching goal of this project is to understand the mechanism by which fatty acid synthase (FASN) activity regulates the expression levels of the oncogenic c-met receptor tyrosine kinase in prostate cancer. My earlier work identified a connection between FASN activity and c-Met protein expression; and more recent data from this reporting period strongly suggests FASN-derived palmitate is required as a post-translation modification to c-Met in order to maintain its stability and trafficking to the plasma membrane. Conclusive evidence for this regulatory mechanism would implicate fatty acid synthase activity and/or palmitoylation as unique therapeutic targets for reducing c-Met expression.

The Specific Aims for this project include:

Aim 1: To determine 1) if c-Met is itself palmitoylated, and if so, at which cysteine residues and 2) if this modification is required for trafficking of c-Met

Aim 2: To investigate a possible correlation of fatty acid synthase with c-Met expression in prostate cancer tissue samples and to investigate a possible causal link between FASN overexpression and c-Met driven prostate cancer using *in vivo* animal models

Body

The work detailed in this project is a continuation of initial findings that determined that inhibition of FASN activity reduced c-Met protein levels. Given that c-Met is an important promoter of prostate cancer metastasis, we sought to elucidate the mechanism by which FASN activity maintains c-Met expression. Our preliminary findings suggest palmitoylation, either of c-Met itself or of other proteins regulating its stability, is involved. In this project, I have detailed experiments to determine if c-Met is palmitoylated and how palmitoylation regulates its stability. In the last funding year, I have made significant progress on the experiments proposed in Aim 1, as will be discussed below.

Determined c-Met to be a palmitoylated protein

I have used both an acyl-biotin exchange technique (data not shown) and a click-chemistry based palmitate-labeling protocol to provide convincing evidence that c-Met is actually palmitoylated (Fig. 1A, B, and C). This evidence has been established in multiple cell lines including DU145 and PC3 prostate cancer cell lines. The binding of labeled palmitate (Az-Palm) to c-Met is sensitive to reducing agents (NH₂OH) indicating the linkage is via a thioester bond at a cysteine residue (Fig. 1A). In addition, labeling with an equal but alternate palmitate, alkyne-palmitate (ODYA), can be prevented in the presence of the palmitoylation inhibitor 2-bromopalmitate (2BP) at time points prior to loss of significant c-Met protein which supports the specificity of labeling (Fig. 1B). I was further able to determine that c-Met is palmitoylated upon synthesis in its 170kd precursor form, most likely in the endoplasmic reticulum (ER), and then remains

palmitoylated as it is cleaved to the mature 140kd form in the Golgi prior to being trafficked to the plasma membrane. Using cycloheximide (CX) to inhibit protein synthesis, brefeldin A (BF) to inhibit transport of protein from the ER to the cis-Golgi, or monensin (MN) to inhibit protein transport from the trans-Golgi to the plasma membrane; we were able to determine that only newly synthesized c-Met becomes labeled, that the 170kd precursor becomes labeled prior to entering the cis-Golgi, and that palmitoylation is maintained without reaching the plasma membrane (Fig. 1C). We plan to perform additional experiments to determine if ligand activation modifies the kinetics of c-Met palmitoylation. This report would be the first evidence for c-Met palmitoylation or palmitoylation of any oncogenic receptor tyrosine kinase. Palmitoylation is known to regulate the activity of a number of other kinases and GTPases that are major influences on prostate cancer growth and metastasis. Samples are currently being analyzed by mass spectrometry at University of South Alabama proteomics facility to conclusively identify the cysteine residue/s linked to palmitate. Future work within this subaim will rely on the information provided by the mass spectrometry analysis. The results will allow me to make mutations at the palmitoylated cysteine residue/s in order to examine the specific effects of c-Met palmitoylation on prostate cancer growth, invasion, and metastasis rather than being limited to the use of general palmitoylation inhibitors.

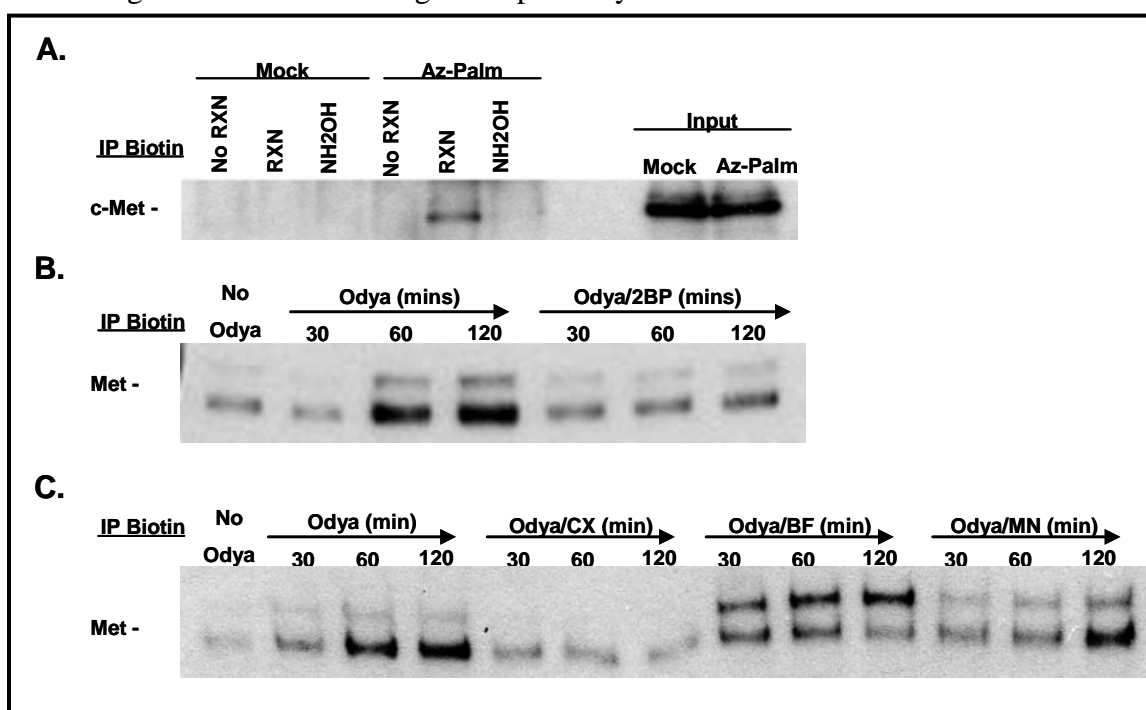


Figure 1. Newly synthesized c-Met is palmitoylated in the Golgi in its 170kd precursor form. For each panel, a Click-chemistry-based technique was used to biotin label and immunoprecipitate palmitoylated protein. A.) DU145 prostate cancer cells were incubated with azide-linked palmitate or mock control for 5 hours. Equal protein for each set was separated for a Click reaction (RXN) or not (No RXN) to link biotin to palmitate having been reduced (NH₂OH) to remove palmitate prior to the reaction as a control or not (RXN). Identification of c-Met as a palmitoylated protein is shown by Western blotting. Relative input levels for mock and the labeled sample are shown. B.) DU145 cells were incubated with an alkyne-linked palmitate (Ody) or without (No Ody) for the indicated times in the presence or absence of 100 μ M 2BP. Levels of palmitoylated c-Met are demonstrated by Western blotting. C.) As above, DU145 cells were labeled with alkyne-linked palmitate (Ody) or without (No Ody) for the indicated times in the presence of cycloheximide (10 μ g/ml) (CX), brefeldin A (2 μ g/ml) (BF), or monensin (2 μ M) (MN). Levels of palmitoylated c-Met are demonstrated by Western blotting.

Inhibition of palmitoylation leads to the post-translational downregulation of c-Met seemingly through a block in intra- or post-Golgi transport.

In order to determine how inhibition of palmitoylation affects c-Met, we treated DU145 prostate cancer cells with 2BP over time. Western blot analysis revealed 2BP treatment leads to a progressive loss of total c-Met protein (Fig. 2A). We next wanted to analyze multiple levels of expression, including transcription, translation, as well as internalization and degradation, to determine how inhibition of palmitoylation downregulated c-Met. Using real-time quantitative PCR we have shown that transcription rates of c-Met are not influenced by 2BP treatment (data not shown). A Click-chemistry-based technique for detecting newly synthesized protein was used to determine that the rate of protein synthesis remains constant in the presence of 2BP (Fig. 2B). Taken together, these data indicate the downregulation must occur post-translationally. Consistent with this, confocal microscopy (Fig. 2C) and surface biotinylation experiments (data not shown) show that newly synthesized c-Met accumulates in the Golgi and is lost from the plasma membrane at a basal rate. Additional experiments suggest a role for ectodomain shedding in the loss of c-Met from the plasma membrane (data not shown). We hypothesize that preventing c-Met palmitoylation causes a build-up in the Golgi that is ultimately targeted for degradation. Future studies will address the mechanism of degradation.

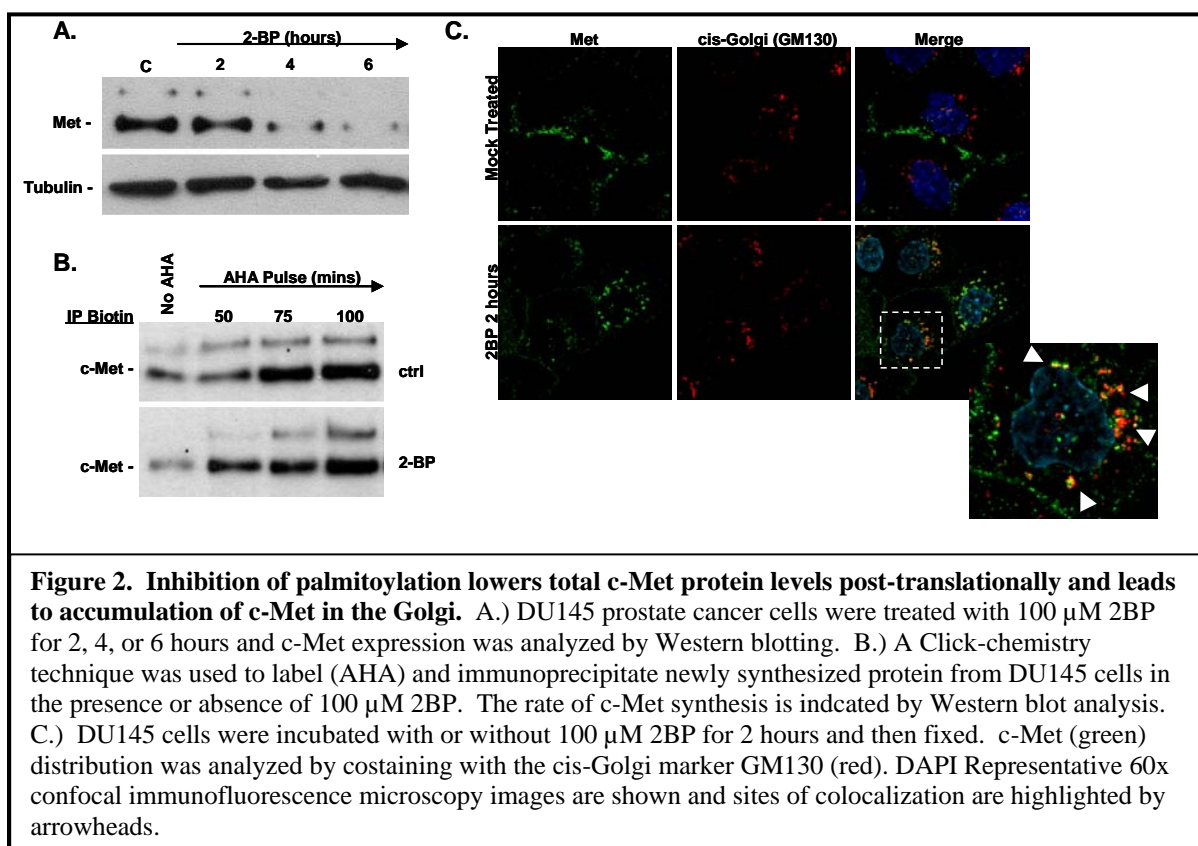


Figure 2. Inhibition of palmitoylation lowers total c-Met protein levels post-translationally and leads to accumulation of c-Met in the Golgi. A.) DU145 prostate cancer cells were treated with 100 μ M 2BP for 2, 4, or 6 hours and c-Met expression was analyzed by Western blotting. B.) A Click-chemistry technique was used to label (AHA) and immunoprecipitate newly synthesized protein from DU145 cells in the presence or absence of 100 μ M 2BP. The rate of c-Met synthesis is indicated by Western blot analysis. C.) DU145 cells were incubated with or without 100 μ M 2BP for 2 hours and then fixed. c-Met (green) distribution was analyzed by costaining with the cis-Golgi marker GM130 (red). DAPI Representative 60x confocal immunofluorescence microscopy images are shown and sites of colocalization are highlighted by arrowheads.

Predoctoral Training Progress

- At the start of this funding year all predoctoral coursework had been completed as indicated in the initial application. I have no new coursework to declare.
- Current training is focused on mentorship, grantsmanship, presentation skills, and laboratory technique.
 - Present and participate in weekly cancer research journal club.
 - Present and participate in weekly departmental seminar series.
 - Continued training by my advisor during weekly research meetings.
 - Mentor students and technicians in the laboratory on a daily basis.
 - Participate in grant writing workshops provided by the LSUHSC-S graduate school.
 - Participated in science advocacy Hill Day event in Washington, D.C. sponsored by American Society for Biochemistry and Molecular Biology (ASBMB).

Reportable Outcomes

Coleman, D.T., and Cardelli, J. c-Met Protein Expression is Regulated by Palmitoylation in Prostate Cancer Cells. Proceedings of the American Association of Cancer Research, Abstract 7446, April 2012.

Key Research Accomplishments

- Determined c-Met to be a palmitoylated protein.
- Determined how blocking c-Met palmitoylation effects the protein's expression.

Conclusions

Over the last several years, increasingly compelling evidence has been published showing that the c-Met receptor is an important therapeutic target for prostate cancer (refs). The data acquired throughout the last reporting year have identified inhibition of palmitoylation, directly, or through inhibition of FASN activity, as a potential therapeutic strategy for downregulating the Overexpression of c-Met often observed in prostate cancer and especially prostate cancer bone metastases. From the data acquired over the last year, our laboratory's model has c-Met being palmitoylated as a precursor in the ER and this fatty acid attachment is required for its stability and proper trafficking out of the Golgi toward the plasma membrane. Blocking palmitoylation causes an accumulation in the Golgi and eventual degradation through a yet to be determined pathway. As far as we are aware, this is the first evidence for the palmitoylation of an oncogenic receptor tyrosine kinase. Future work will be aimed at 1.) identifying the specific cysteine residue/s palmitoylated, 2.) better defining the intracellular site and kinetics of c-Met palmitoylation and degradation with higher resolution, and 3.) establishing proof of principle data in animals models using wt or palmitoylation-defective mutants of c-Met as laid out in the proposal. In addition, continued predoctoral training will emphasize the skills listed above.

Personnel Receiving Salary from W81XWH-11-1-0570

1. David T. Coleman

Appendix

AACR Annual Meeting Abstract 7446

c-Met Protein Expression is Regulated by Palmitoylation in Prostate Cancer Cells
Department of Microbiology and Immunology and The Feist-Weiller Cancer Center
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David T. Coleman and James A. Cardelli

Prostate cancer (PCa) is the most common noncutaneous neoplasia diagnosed in men and represents roughly 10% of cancer-associated mortalities. The lethal phenotype of PCa is primarily characterized by progression of tumor cells to androgen-independence and metastasis. Organ-confined PCa is curable with surgery and/or radiation therapy, however, as the disease becomes metastatic, the likelihood of survival becomes minimal. Key influences in the progression to metastasis are growth factor receptors including the receptor tyrosine kinase c-Met. Numerous bodies of evidence strongly link aberrant c-Met signaling with causation and/or progression of prostate cancer. Overexpression of c-Met can lead to activation in a ligand-independent manner and has been identified as a common mechanism of resistance to RTK-targeted therapy. In addition, multiple reports have identified the enzyme fatty acid synthase (FASN) as being commonly overexpressed in prostate cancer, and that this aberrant expression is an early event that becomes more pronounced with aggressive androgen-independent and metastatic disease. FASN is the sole enzyme responsible for *de novo* synthesis of the 16-carbon saturated fatty acid palmitate. In cancer, *de novo* lipids are more selectively partitioned into lipid rafts as phospholipids as well as utilized for post-translational acyl-modifications of signaling proteins. Previous findings have led us to identify a novel mechanism by which FASN activity regulates c-Met expression. Our work has determined that inhibition or shRNA knockdown of FASN results in a post-translational downregulation of already synthesized c-Met protein. This downregulation is prevented by the addition of exogenous palmitate.

Here we provide evidence that inhibition of palmitoylation, using the palmitate analog 2-bromopalmitate, lowers total c-Met levels. This protein loss occurs post-transcriptionally and is associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization pathways, as well as surface biotinylation studies, our findings suggest the inhibition of palmitoylation reduces the stability of newly synthesized c-Met as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a click-chemistry based palmitate-labeling protocol have provided evidence suggesting c-Met itself is palmitoylated. From these data, we hypothesize that c-Met requires palmitoylation along a biosynthetic route to promote its stability and trafficking to the cell surface. In the absence of palmitoylation, c-Met accumulates within the Golgi and is disposed of through a yet to be determined degradation pathway. Our findings potentially reveal a novel mechanism for restricting the expression of c-Met as a means of preventing prostate cancer invasion and metastasis.